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Choosing a healthy mate: sexually attractive traits as reliable indicators of current disease status in house mice

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ABSTRACT

Social interactions are critical for reproduction in many animals. Since several pathogens are transmitted by social contact, females searching for mating partners should select males that can signal being healthy. Not all signals, however, may be reliable, since males from a number of species can overcome behavioural symptoms of infection when mating opportunities are available. Here, we manipulated sickness status of male house mice (*M. musculus domesticus*) by administering an immune challenge (lipopolysaccharide, LPS) and studied the consequences of this manipulation on two signals that function in mate attraction in this species: ultrasonic vocalizations (USVs) and Darcin (a urinary protein). Additionally, we quantified female visits to immune-challenged and control males, and the males' plasma testosterone levels. LPS-injected males had lower Darcin and lower regular ultrasonic syllable production than control-injected males, while producing a larger number of high frequency ultrasonic syllables. We conclude that immune-challenged male mice presented with a receptive female cannot maintain the production of sexually attractive signals. Females might use some of these cues when making mating decisions, since they spent significantly less time near LPS-injected males. Testosterone was reduced in LPS-injected males and could be a unifying mechanism downregulating both of the traits quantified. Darcin and USVs produced in the context of courtship may therefore function as reliable indicators of current health status.

KEYWORDS

Darcin; disease; honest signalling; major urinary proteins; testosterone; ultrasonic vocalizations

INTRODUCTION

Choosing a sick social or mating partner is risky. An individual risks disease or parasite infection, or reduced benefits provided by the partner due to its inferior health. Mechanisms for the recognition and avoidance of parasitized or sick conspecifics should therefore reduce the probability of exposure and be beneficial. Indeed, animals of several species are able to distinguish between parasitized and healthy conspecifics (reviewed in Beltran-Bech & Richard, 2014). While this recognition may be beneficial for healthy individuals, it could be disadvantageous for sick animals looking to pass on their genes when the probability of survival is perceived to be low (terminal investment hypothesis; see Clutton-Brock, 1984).

Animals use different types of communication to gain information about potential partners and this is especially critical for mate choice. Females should pay close attention to signals that allow them to find potential mates. If the signals also indicate male quality, then females can use these signals to make qualitative choices amongst available suitors. In sexually reproducing species, honest signalling of condition and health is considered to be of prominent importance for female choice of a mating partner (Andersson, 1994). Not all signals, however, are necessarily honest, since males from a number of species are able to overcome behavioural symptoms of infection when mating opportunities are available (summarized in Lopes, 2014).

Here, we study the consequences of experimental manipulation of sickness on two signals that function in mate attraction and mate choice in house mice (*M. musculus domesticus*): ultrasonic vocalization and urinary proteins.

Females spend more time near male mice that are able to produce ultrasonic vocalizations (Pomerantz, Nunez, & Bean, 1983) and are attracted to the playbacks

of song-like vocalizations emitted by adult males (Hammerschmidt, Radyushkin, Ehrenreich, & Fischer, 2009; Musolf et al., 2010). Another important route of communication for rodents consists of olfactory cues deposited in the urine (Hurst & Beynon, 2008). Proteins with signalling functions found in mouse urine include major histocompatibility complex (MHC), major urinary proteins (MUPs) and their volatile ligands (Hurst & Beynon, 2008). MUPs consist of the majority of the protein content found in male mouse urine (Finlayson, Potter, & Runner, 1963; Humphries, Robertson, Beynon, & Hurst, 1999), and their level is sexually dimorphic with males having a three to four times higher concentration of urinary MUPs than females (Beynon & Hurst, 2004). MUP expression patterns provide different types of information, such as genetic and individual identity (Cheetham et al., 2007; Sherborne et al., 2007), but one particular MUP stands out in terms of mate attraction potential: Darcin. Through a series of experiments, Roberts and colleagues (2010) demonstrated that Darcin alone is responsible for the preference that female mice have for male versus female urine, and that females are not attracted to male urine containing low levels of Darcin.

While both vocalizations and Darcin serve the function of attracting females, females might extract other information from them when selecting a potential mate. Besides functioning as a way to assess genetic quality and compatibility of a male (Hoffmann, Musolf, & Penn, 2012; Hurst, 2009; Hurst et al., 2001), these types of signals could work to reflect a male's current condition, for example, his disease status. From the female's perspective, detecting and avoiding mating with sick males should be important for several reasons, including: decreased likelihood of future male services (e.g. parental care, territorial defence; Andersson, 1994); poor male genetic quality (e.g. high susceptibility to disease; Hamilton & Zuk, 1982); and higher risk of disease

infection (Able, 1996) and consequential loss of pregnancy (Aisemberg et al., 2010). Both acoustic signalling and urinary protein production are thought to be costly (Nelson, Colson, Harmon, & Potts, 2013; Ryan, 1988). Thus, from the perspective of the sick male, signalling can become dangerous if the energy necessary to produce the signal competes with the energy necessary to recover from the infection. However, if males can temporarily overcome symptoms of infection to attract and mate with females, this risk might be compensated for by successful fertilization (Lopes, 2014).

Immune challenges can disrupt reproductive physiology (for a review, see Tomaszewska-Zaremba & Herman, 2009), with consequences for testosterone production (Boonekamp, Ros, & Verhulst, 2008). Thus, when responding to an infection, animals may experience reduced levels of testosterone. If we consider that many secondary sexual traits, including ultrasonic vocalizations and Darcin production, are known to be under androgenic influence (Knopf, Gallagher, & Held, 1983; Nunez, Nyby, & Whitney, 1978), it becomes plausible that, when animals are infected, trait production may be affected as a consequence of the associated decrease in testosterone. Therefore, testosterone is a likely candidate to link immune activation to changes in production of Darcin and ultrasonic vocalizations.

In the current study, we tested whether sickness status impacted the amount of Darcin and the number of ultrasonic syllables produced by wild-derived male house mice (*Mus musculus domesticus*) when exposed to a female in oestrus. Manipulation of sickness status was done by administering an inflammatory challenge using lipopolysaccharide (LPS) injections. When faced with deteriorating health conditions, males may attempt to invest in reproduction over recovery, and produce sexually attractive signals. We predicted that, if this system were susceptible to dishonesty,

the easier signal for mice to manipulate would be vocalizations, as this signal is potentially under voluntary control (Seyfarth & Cheney, 2010). It should be harder for mice to manipulate the levels of Darcin expression and we thus predicted a decrease in Darcin levels during an inflammatory challenge. Given its important role as a modulator of the sexually attractive signals quantified here (Knopf et al., 1983; Nunez et al., 1978), we also measured testosterone in experimental males. We used the time females spent near sick versus control males as a proxy for attractiveness.

METHODS

Ethical Note

Animal use and experimental design were approved by the Veterinary Office Zürich, Switzerland (Kantonales Veterinäramt Zürich, no. 88/2014).

Animals

The experiments were carried out with house mice (*Mus musculus domesticus*) in an animal facility at the University of Zürich. Experimental animals were born in the lab and represented F1 to F3 descendants of wild house mice captured in the vicinity of Illnau, near Zürich. Animals were kept under standardized laboratory conditions at a temperature of 22°C ± 3°C with a relative humidity of 50–60% and on a 14:10 light:dark cycle with a 1 h sunrise (6:00 to 7:00) and dusk (19:30 to 20:30) phase at the beginning and end of the light phase (white light started at 6:30 and turned off at 20:30). We tested 21 sexually mature, but non-breeding brother pairs (mean age ± SE = 60 ± 1 days), and 21 sexually mature non-breeding females (mean age ± SE = 105 ± 6 days).

In our facility, male and female siblings are separated after weaning at 23 days of age and housed in single-sex groups in standard Makrolon Type III cages. Between 5-6 days before the start of an experimental trial, a pair of male siblings was randomly chosen from one of these cages and each was placed in a separate cage with an unrelated, unfamiliar, sexually mature, but sterile female (hybrid obtained by mating mice from different chromosomal races as part of a separate experiment). The vocalizations emitted by the pair were observed for 1h, using an Avisoft ultrasound-microphone (Ultrasound Gate CM16/CMPA) connected to a single-channel recording device (Ultrasound Gate 116Hb, Avisoft Bioacoustics, Berlin, Germany). As it has been found that not all mice vocalize when tested (Christine Pfeifle, personal communication), male pairs were only used further if we observed vocalizations being emitted by both during this first hour in the presence of a female. These males were housed with the sterile female for at least 5 days prior to the experiment. Given that these males were separated from their siblings before any signs of aggression, we have no reasons to believe that dominance relationships had been formed at this time. In addition, the mass of male siblings at the start of the experiment was not significantly different (paired *t*-test, $P = 0.38$).

All animals were provided with food (laboratory animal diet for mice, Provimi Kliba SA, Kaiseraugst, Switzerland) and water *ad libitum*. The contents of the cages consisted of standard bedding material (Lignocel Hygienic Animal Bedding, JRS), as well as shredded paper towel and an empty toilet paper roll.

Experimental Setup

Recording box

All experiments were conducted in the same room where the animals were housed after weaning, and we used a recording box as described in von Merten, Hoier, Pfeifle, and Tautz (2014), with a few adaptations for our experimental purposes. Briefly, our box consisted of three separate compartments, located side by side. The middle compartment was connected to the outer two via a round window (6.5 cm of diameter) on each side, covered with mesh-wire (spacing of the wire was 1 mm). Mice in the outer compartments could thus interact with the mouse in the central compartment through these windows. A round piece of PVC (same material as rest of the box) could be placed over the communication windows and tightly closed to prevent visual, physical and at least reduce olfactory and acoustic interactions.

During the test, mice had food and water *ad libitum* available in each compartment, which further contained standard bedding material and one empty toilet paper roll to allow for hiding and resting.

Over each compartment, one ultrasound-microphone (Ultrasound Gate CM16/CMPA) was fixed to a metal rod. The microphones were positioned at 25 cm from the wall and 30 cm above the ground of the experimental box. Each microphone was connected to a single-channel recording device (Ultrasound Gate 116Hb, Avisoft Bioacoustics, Berlin, Germany). Over the course of the experiment, we recorded continuously at a sampling rate of 250 kHz and a depth of 16 bits. The recorder software was programmed to save the recordings in 10 min time bins. The positioning and the setup of the microphones reduced the probability of recording USV from the adjacent animal (von Merten et al., 2014). To be conservative, we removed recordings captured simultaneously by two adjacent microphones.

To record the behaviour of the animals inside the recording box, we used digital video cameras sensitive to low lux (Sony), recording from the top of the compartments under red light.

Timeline

On the morning of the experiment, at 11:30, we recorded the individual body weight of both male siblings (Sartorius scale, BL1500S) and placed each of them in one of the outer compartments of the recording box (Fig. A1). This placement, as well as injection treatment, had been randomly assigned prior to weighing. At this time, the communication windows were closed. Males were placed in the recording box several hours before the onset of the experiment, since males of some laboratory strains vocalize when separated from a female (Yang, Loureiro, Kalikhman, & Crawley, 2013) and we wanted to avoid this effect. At approximately 17:00, we chose a female stimulus, weighed her and placed her in the middle compartment. The female stimulus was unrelated and unfamiliar to the focal test males. Receptive females were chosen based on oestrous stage. This was assessed by vaginal tissue inspection following the visual method described in Byers, Wiles, Dunn, and Taft (2012), which was proven to be reliable for assessing female receptiveness (proestrus and estrus stage).

The female was only placed in the recording box at this time to allow for a more accurate estimate of oestrus (closer to the start of the recordings), while still allowing the female to habituate to the recording box before the start of the experiment.

At 19:20, both males were weighed again and at approximately 19:30 they were injected with either lipopolysaccharides (LPS from E. coli, Serotype 0111:B4, Sigma-Aldrich #L4391) dissolved in saline (Sodium Chloride solution 0.9%, Sigma-Aldrich

#S8776) or saline alone. The dose of LPS was 0.6 µg/g of body weight (approximately 15 µg per animal). LPS is a component of the cell wall of gram-negative bacteria and it is frequently used in experimental settings aimed at inducing sickness behaviours in animals (Lopes, 2014). The advantage of this approach over an actual infection is that all the effects observed are due to the host's response, as opposed to additional effects induced by the infectious agent. The dose used was established from a pilot study and the range of doses to be tested in the pilot was determined based on previous studies done in *Mus domesticus* (Downs et al., 2012; Downs et al., 2013). At 3 h after injection (22:30), the communication windows were opened by manual removal of the PVC cover and animals were allowed to interact through the mesh-wire. The behaviours and vocalizations of all mice were recorded from 20:30 (start of dark phase) until 6:30. The next day, mice were weighed once more at 11:00 (with the exception of some males that were removed already at 07:30 for gene expression analyses, see below) and kept singly in Type II cages, until they were euthanized by CO_2 inhalation within approximately 1 h after the end of the experiment. The test compartments were cleaned with 70% ethanol, allowed to dry and re-furnished with new bedding, toilet roll, food and water.

Behaviour

For each mouse, we quantified behaviours for 30 min at three time points: 21:00 (before the communication windows were opened - window 'Closed'), 23:00 (window 'Open short-term') and 3:00 (window 'Open long-term'). During each of these periods, we measured the amount of time males spent inactive, as reduced activity is a common symptom of sickness behaviour in mice. To have a measure of male interest on the female, we measured the amount of time spent in contact with the communication window. To assess whether the female showed signs of preference

for one male over the other we quantified the amount of time spent in contact with each communication window. In both instances, contact with the window was defined as having any body part touching either the PVC piece that closed the window, the mesh-wire in the window or the region immediately underneath the window. The time points chosen allowed us to analyse if the females had an initial side preference (before windows were open), whether a preference existed soon after windows were open and whether a preference was established with further exposure to the males. A later time point could have missed the more acute phase of the infection and also may have caused the females to lose their preference due to habituation (Hammerschmidt et al., 2009).

Vocalization Analysis

The recorded vocalizations were visualized using Avisoft SASLab Pro Version 5.2, using the following settings: Fast Fourier Transform (FFT)-length of 512 points, 100% frame size with FlatTop window and a time window overlap of 50%. The number of syllables emitted every 10 min was counted by hand. Syllables were separated into two classes, according to their frequency ranges: 30 to 110 kHz (within the normal frequency range described for mice, reviewed in Portfors, 2007), here forth called "regular syllables", and an unusual and rare syllable category higher than 110 kHz, here forth called "high syllables". Studies in rats have suggested that syllables of different frequencies are emitted under different circumstances having positive or negative valences (reviewed in Brudzynski, 2013).

Gene Expression

To explore the impact of the treatments on liver production of proteins that have been shown to serve a role in attraction of females when deposited in the urine, we

quantified mRNA production of Darcin in the liver. Given that hepatic and urinary MUP protein levels in adult mice generally follow the pattern of mRNA expression in the liver (Isseroff, Sylvester, & Held, 1986; Ramirez, Luque, Ornstein, & Becu-Villalobos, 2010; Giller, Huebbe, Doering, Pallauf, & Rimbach, 2013), assessment of Darcin via quantification of hepatic gene expression should provide a good overall picture of Darcin levels. For this, we used a subset of the males from the main experiment (*N* = 10 brother pairs). At 12 h after the injection (07:30 the following morning), we took the males from the recording box, weighed them and euthanized them by CO_2 inhalation. Males were decapitated and trunk blood was collected into 1.5 mL tubes and preserved on ice until centrifugation, after which the plasma portion was collected into separate tubes. The plasma was used to quantify testosterone (described in section *Testosterone*). We extracted the liver, which was immediately frozen in dry ice and kept at -80 ^{o}C until further use. The time point for liver sampling was chosen based on experiments showing that LPS-induced symptoms last for less than 24 h (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008), and that the effects of infection on the number of females attracted to scent marks of male mice can still be observed at 3 to 5 days post-infection (Zala, 2004).

RNA Isolation, Purification and Reverse Transcription

Total RNA extraction was performed according to the manufacturer's instructions, including the genomic contamination step (NucleoSpin RNAII, Macherey-Nagel, Düren, Germany), with final dilution of RNA in RNAse-free water. Quantification of RNA was done via spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific). The RNA samples were reverse transcribed to cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany), according to manufacturer's instructions.

289 *Quantitative Real-time PCR (qRT-PCR)*

290 Primers for the gene of interest (Darcin) and for the housekeeping genes (TATA Box
291 Binding Protein - TBP, and Eukaryotic Translation Elongation Factor 1 Alpha 1-
292 EEF1A1), were designed with the aid of Primer-BLAST based on the published
293 sequences for *Mus musculus* (available in GenBank). Primer sequences were (5' to
294 3'): Darcin forward (F): GGCCCGAGAATGAAGAATGG; Darcin reverse (R): GCA
295 GATCACAGAACTTCTTACTGG; TBP-F: TTGACCTAAAGACCATTGCACTTC; TBP-
296 R: TTCTCATGACTGCAGCAA A; EEF1A1-F: TCCACTTGGTCGCTTTGCT, and
297 EEF1A1-R: CTTCTTGTCCACAGCTTTGATGA. Amplification of primer dimer,
298 unwanted products and genomic DNA was controlled for by running total RNA
299 controls from each individual. These samples always resulted in differences in at
300 least 7 cycles of the C_t (threshold cycle) values compared to the cDNA samples.
301 qRT-PCR was performed in triplicate for each cDNA sample for each gene according
302 to the manufacturer's instructions for MESA GREEN qPCR Mastermix Plus for SYBR
303 (Eurogentec, Cologne, Germany). After checking for primer specificity by visual
304 inspection of the melting curves, we used the C_t values to compare expression
305 between control and LPS-injected animals. The reference genes used to normalize
306 mRNA levels among samples were TBP and EEF1A1 after verification that treatment
307 did not affect their levels (*t*-test, $P > 0.05$). Normalization was done for each
308 individual by dividing expression values for the gene of interest by the geometric
309 mean of the expression of the housekeeping genes for the corresponding sample.

310 *Testosterone*

311 The testosterone assay was performed using a commercial ELISA kit (ADI-900-065,
312 Enzo Life Sciences, Lausen, Switzerland) following the protocol described in de

313 Peyster et al. (2008). All samples were run in duplicate on a single plate and intra-
314 assay variation was 9.3%.

315 *Statistical Analysis*

316 Statistical tests were carried out using R version 3.1.2 (R Core Team, 2014) with the
317 add-on packages lme4 (Bates, Maechler, Bolker, & Walker, 2014) and glmmADMB
318 (Fournier et al., 2012; Skaug, Fournier, Bolker, Magnusson, & Nielsen, 2015).

319 To test whether the time points at which behavioural data was collected (i.e. window
320 'Closed', 'Open short-term', 'Open long-term') interacted with injection treatment to
321 affect time females spent at each males' communication window, and time males
322 spent inactive, we used linear mixed effects models, including the factors time point,
323 injection treatment and the interaction of the two. The model was the same to test the
324 time males spent at window, only this response was better fitted by using a
325 generalized linear mixed effect model with Poisson error distribution. These models
326 included a random effect of male identity nested to female identity. To further
327 investigate the time of night at which female visitation differed for control and LPS we
328 used Tukey Contrasts.

329 To test whether mass changed over the three time points of measurement (i.e. 'start
330 of experiment', 'at injection', 'end of experiment') and whether this change interacted
331 with injection treatment, we used linear mixed effects models, including the factors
332 time point, injection treatment and the interaction of the two. Here we also included a
333 random effect of male identify nested to female identity.

334 To test whether injection treatment affected the number of syllables produced (high
335 and regular tested separately), and testosterone secretion, we used generalized
336 linear mixed effects models fitted by a Poisson error distribution. To test for an effect

of injection on Darcin gene expression we used a linear mixed effect model. These models included a main effect of injection and a random effect of female identity. Since pairs of siblings were tested against the same females, this random effect encompasses the effect of brother relatedness.

Overdispersion of the data fitted by a Poisson error distribution was accounted for by including an observation level random effect (OLRE; Harrison, 2014), unless overdispersion seemed to be associated with excessive number of zeros (zero-inflation), in which case we used zero-inflated models instead. Unless otherwise stated, models were fitted with using normal error structures after verification of the normality and homoscedasticity of the residuals. The significance of the predictor variables in the models was assessed using *F* tests for the linear models and Wald χ^2 tests for the generalized linear mixed models (following the recommendations in Bolker et al., 2009).

In all figures, data are represented as means ± 1 standard error of the mean (S.E.M.).

RESULTS

Males injected with LPS spent significantly more time inactive than their control-injected siblings ($F_{1,20}$ = 332.028, P < 0.0001). Time of night had a significant effect on time inactive ($F_{2,80}$ = 11.92, P < 0.0001), with males spending less time inactive after the communication windows were opened (Fig. 1a). The interaction between injection treatment and time of night was not significant (P > 0.05). The time males spent visiting the window that provided access to the female was significantly affected by injection treatment (χ^2 = 75.88, P < 0.0001), time of night (χ^2 = 23.92, P < 0.0001), and the interaction of the two (χ^2 = 6.33, P = 0.042). As can be seen from

Fig. 1b, the difference between control and LPS was larger at the two time points after opening of the windows.

Male mass decreased significantly over time ($F_{2,80}$ = 176.01, P < 0.0001; Fig. A2), but was not affected by injection treatment or the interaction of injection treatment and time of night (P > 0.05).

The number of regular syllables produced by males was significantly affected by injection treatment (χ^2 = 25.13, P < 0.0001), with control males emitting more syllables than LPS-injected siblings (Fig. 2a). High syllables were also affected by injection (χ^2 = 31.36, P < 0.0001), only this time in the opposite direction, with LPS-injected males producing more of these syllables than control males (Fig. 2b).

Darcin expression in the liver was significantly lower in LPS-injected animals as compared to controls ($F_{1,9}$ = 71.99, P < 0.0001; Fig. 3a). Testosterone levels were also significantly lower in LPS-injected animals as compared to controls (χ^2 = 11.87, P < 0.001, Fig. 3b).

The time females spent visiting the windows that connected them to the male compartments was affected by injection treatment of the male ($F_{1,20}$ = 5.25, P = 0.033), time of night ($F_{2,80}$ = 22.88, P < 0.0001), and the interaction of the two ($F_{2,80}$ = 4.83, P = 0.01). The females transitioned from spending the same amount of time near either communication window (Closed and Open short-term, P > 0.05, Tukey contrasts) to spending significantly more time at the windows leading to the control males than to their LPS-injected brothers (Open long-term, P = 0.0022, Tukey contrasts, Fig. 4).

DISCUSSION

Wild-derived male house mice (*Mus musculus domesticus*) undergoing a simulated infection decreased the expression of Darcin in the liver and the overall number of regular ultrasonic syllables produced in the presence of an unrelated receptive female. Since both traits are known to play a role in female attraction (Pomerantz et al., 1983; Hammerschmidt et al., 2009; Musolf, Hoffmann, & Penn, 2010; Roberts et al., 2010), we conclude that males of this species cannot sustain the production of sexually attractive signals while responding to an infection.

Male house mice injected with lipopolysaccharides (LPS) exhibited symptoms of sickness behaviours, showing a much greater degree of inactivity than their control-injected siblings, which is the expected outcome from this type of manipulation (Hart, 1988). These males also showed significantly less interest than control-injected siblings in engaging with the females through the communication windows, which is consistent with a previous finding in a lab strain of *M. musculus* (Weil, Bowers, Pyter, & Nelson, 2006) where LPS injection eliminated mating behaviour. However, not all studies observe reduced male interest (summarized in Lopes, 2014). For example, in rats, mating behaviour was maintained in immune-challenged males (Avitsur & Yirmiya, 1999; Yirmiya, Avitsur, Donchin, & Cohen, 1995). A different study using the same lab strain of mice as Weil and colleagues (2006) found males infected with the intestinal nematode *Heligmosomoides polygyrus* to be equally receptive to females as uninfected males (Ehman & Scott, 2002). A recent experiment on wild-derived *M. musculus* showed no difference in sexual motivation between *Salmonella*-infected and healthy males (Zala, Bilak, Perkins, Potts, & Penn, 2015). Since our measure of interest for females was time spent at the female window, our results are not directly comparable to measures of other mating behaviours (such as mounting, intromissions and ejaculation). In these previous studies, males had direct contact

409 with the female and thus the opportunity to copulate with her, which should provide
410 greater motivation to display sexual behaviours. Males in the current study were
411 separated from the females by a mesh covered window, which may account for the
412 lower interest observed in LPS-injected males. Contrary to some previous studies,
413 LPS injection had no effect on male mass. All males lost a large amount of mass
414 before injection, potentially due to handling and/or acclimation to a new environment
415 (the recording box). This initial mass loss may have hindered the potential effects of
416 LPS injection on mass. A similar effect has been observed in zebra finches (Lopes,
417 Adelman, Wingfield, & Bentley, 2012).

418 Of the two signals being quantified, we predicted that syllable production would be
419 the most amenable to manipulation by the males, while Darcin gene expression
420 would not be subject to manipulation. Contrary to this expectation, both syllable
421 production and Darcin were drastically affected by LPS injection. A previous study in
422 mice found mRNA encoding major urinary protein (MUPs) as well as MUP urinary
423 levels to be decreased after 20 days of infection with *Schistosoma mansoni* (Isseroff
424 et al., 1986). However, the same study found very minor changes in MUP expression
425 when the mice were injected with turpentine and the authors concluded that changes
426 in MUP were not occurring due to inflammatory processes. Our results, however,
427 showed a strong effect of LPS-injection on Darcin, suggesting that the cascade of
428 processes triggered during inflammation affects, at some point, the expression of
429 Darcin in the liver. It seems, therefore, that inflammation by infection, but not
430 inflammation induced by chemical irritants, is able to elicit responses that ultimately
431 impact Darcin levels. This is the first time that Darcin gene expression has been
432 shown to change this rapidly (within 12 h) in response to a non-replicating antigen.

In our study, the host's response to an immune challenge was sufficient to lead to a decrease in circulating testosterone. Given that MUP expression is partially under androgenic control (Knopf et al., 1983), the decrease in Darcin gene expression in LPS-injected mice could be associated with changes in testosterone. Indeed, our LPS-injected males had significantly lower testosterone levels than controls. This concurs with Isseroff and colleagues (1986), who observed that *S. mansoni* infected mice had much lower levels of testosterone than the uninfected mice and that administration of testosterone to infected mice restored the level of MUPs. Testosterone supplementation has also been shown to rescue levels of urinary proteins in antigen-treated mice (Litvinova, Kudaeva, Mershieva, & Moshkin, 2005). While Darcin and ultrasonic vocalizations represent very different signalling modalities, ultrasonic vocalizations have also been shown to be under androgenic influence (Nunez et al., 1978). Changes in testosterone levels as a consequence of immune activation could thus provide a unifying mechanism for the downregulation of the two sexually attractive signals quantified in the current study. Future studies involving, for example, testosterone supplementation and androgen receptor antagonists, will help further elucidate the importance of testosterone in playing this role.

It is interesting to note that, while the production of "regular syllables" was lower in LPS-injected males, the number of "high syllables" emitted was higher compared to controls. Male mice are thus not entirely muted by LPS injection, opening the potential for continued production of ultrasonic vocalizations to court females while sick. However, as mentioned above, we did not find this effect. These "high syllables" were rare and represented only a small proportion of the total amount of syllables produced by males. In case females in this study were using the number of ultrasonic

458 syllables to make decisions on which male to spend more time with, then
459 attractiveness may lie in the "regular syllables". Since "high syllables" were almost
460 completely absent from the vocalizations produced by control males, one can
461 speculate that they might even be related to a negative/aversive state. In rats,
462 syllables of different frequencies are emitted in aversive versus appetitive situations
463 (reviewed in Brudzynski, 2013). Studies in rats also suggest that the "appetitive calls"
464 induce approach behaviour by other rats, while the "aversive calls" lead to an
465 inhibition of locomotor activity (Wöhr & Schwarting, 2007). Further studies should be
466 carried out to determine the valence of the "high syllables" in mice and the instances
467 in which they occur. If they are only associated with disease, then they could become
468 a tool for disease detection in animal facilities. If they occur more broadly during
469 negative emotional states, then there might be potential to use these types of
470 syllables to assess welfare of laboratory mice.

471 Our results revealed that, after a few hours of exploration, female house mice were
472 able to distinguish amongst sick (LPS-injected) and healthy males, preferring to
473 spend more time close to the latter. Previous studies have shown that females can
474 distinguish between infected and uninfected males, preferring to spend time with the
475 healthy ones. Urine appears to play an important role in how the females make this
476 distinction (Zala, 2004; Kavaliers, 1995; Kavaliers & Colwell, 1995; Klein, Gamble, &
477 Nelson, 1999; Penn, Schneider, White, Slev, & Potts, 1998; Willis & Poulin, 2000;
478 Litvinova et al., 2005). We provide evidence here of an impact of a simulated
479 infection on two sexually attractive signals. Changes in these signals could provide a
480 means by which female mice distinguish sick from non-sick males and establish their
481 preferences. Females should thus account for these signals (or their absence) when
482 making decisions about which males to interact and mate with. We cannot distinguish

which signals females actually used to define their preferences in the current study (particularly given that physical contact may be necessary to detect Darcin; Roberts et al., 2010) or whether other signals may also play a role. However, the two signals we quantified have powerful effects on female preference (Hammerschmidt et al., 2009; Musolf et al., 2010; Roberts et al., 2010) and are likely candidates to modulate the preference that female mice have for healthy over infected males.

In sum, two different types of sexually attractive signals produced by male house mice appear to reliably indicate current health status, as they cannot be maintained once males are undergoing an inflammatory response. We suggest that the decrease in testosterone associated with immune activation may serve as the common modulator regulating both of the signals, but this idea must be assessed by further experimental manipulations. If true, then female house mice may be able to trust any androgen-dependent signal to assess the health of males.

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699 Fig. 1 - Time males injected with either LPS or control spent inactive (a) or at the
700 communication window (b) over the course of the night.

701 Fig. 2 - Number of syllables emitted overnight by males injected with either LPS or
702 control. Two types of syllables were quantified: (a) "regular syllables" between 30
703 and 110 kHz of frequency and (b) "high syllables" over 110 kHz of frequency.

704 Fig. 3 - Gene expression of Darcin in liver (a) and plasma testosterone levels (b) of
705 males at 12 h after injection with either LPS or control.

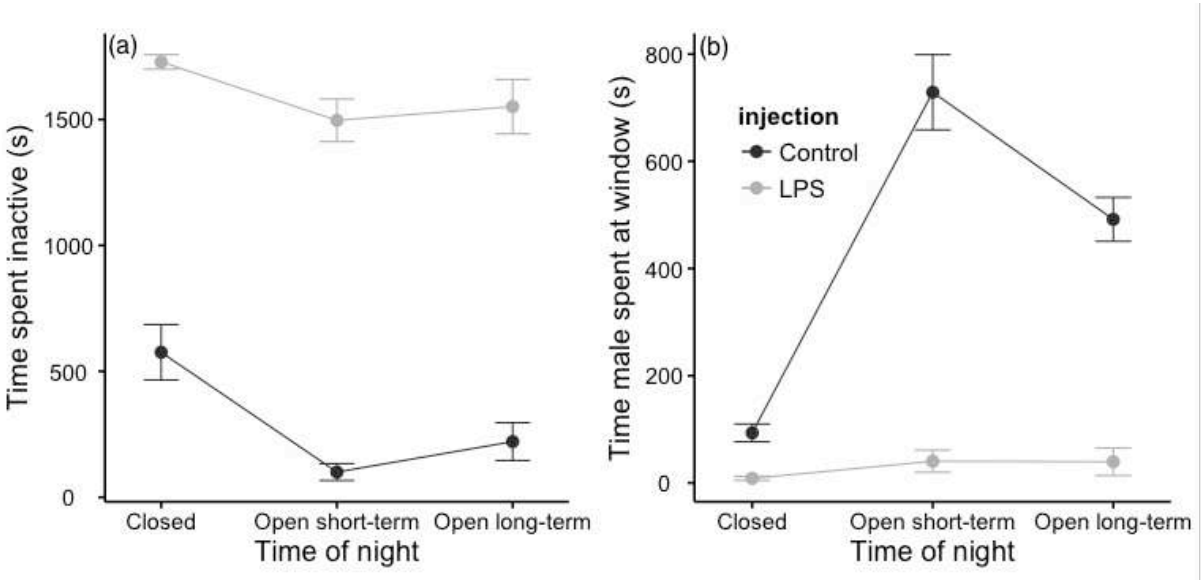
706 Fig. 4 - Time females spent visiting two opposite windows, each connected to
707 separate chambers containing one male injected with either LPS or control, over the
708 course of the night. Asterisk indicates significance at $P < 0.05$ for post-hoc Tukey
709 contrasts.

710 Fig. A1 - Experimental timeline. The communication windows were closed when mice
711 were first place in the recording box. Behaviours were quantified using video
712 recordings and each behavioural measurement took 30 min. Ultrasonic recordings
713 were taken during the entire dark phase. Please find detailed description in the main
714 text.

715 Fig. A2 - Mass of males injected with either LPS or Control over three time points:
716 start of trial; at the time of injection; at the end of the trial. Data are represented as
717 means ± 1 S.E.M.

718

719 Figure 1



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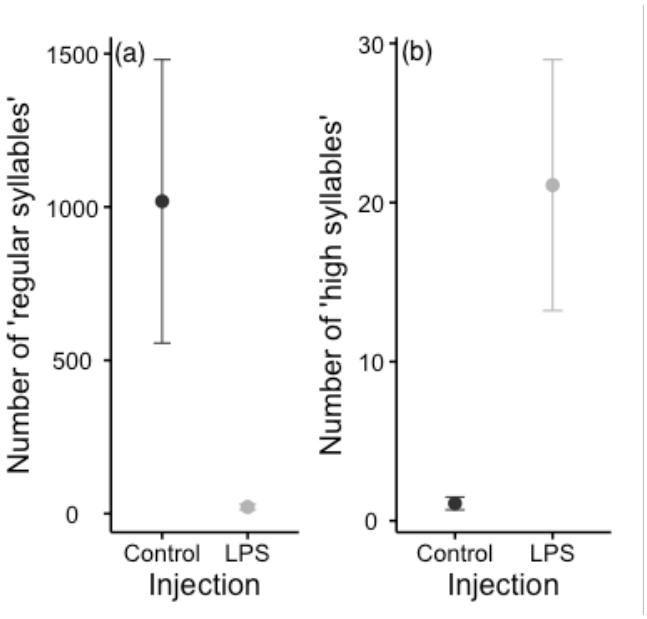
722 Figure 2



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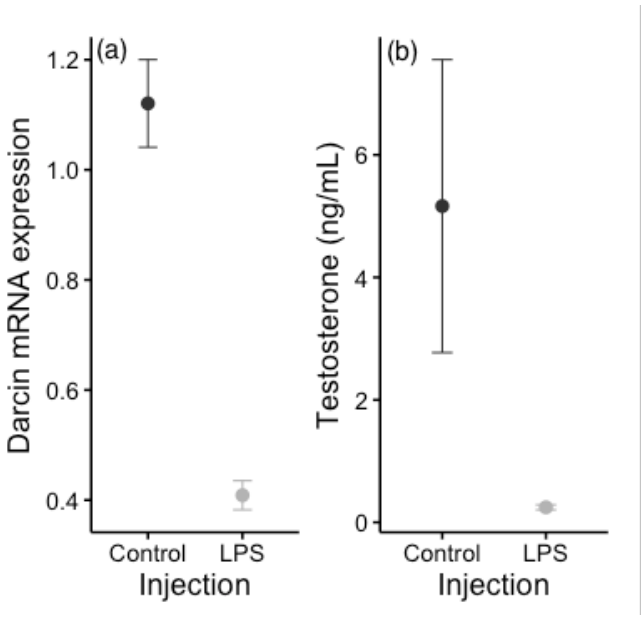
725 Figure 3



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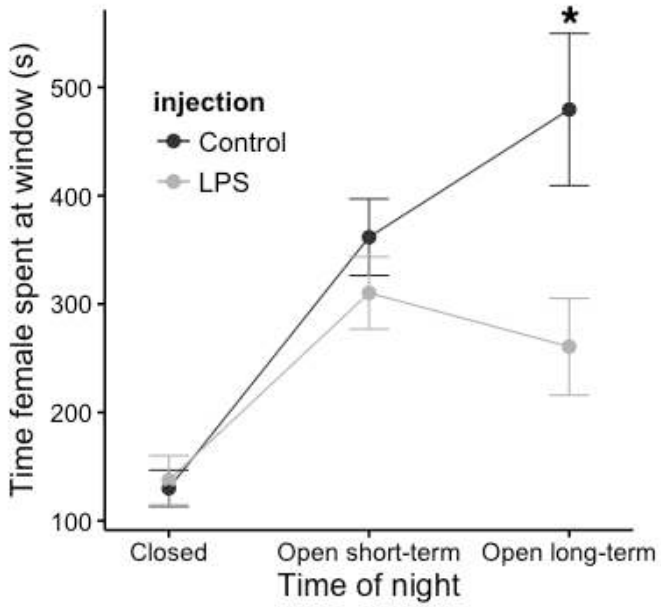
728 Figure 4



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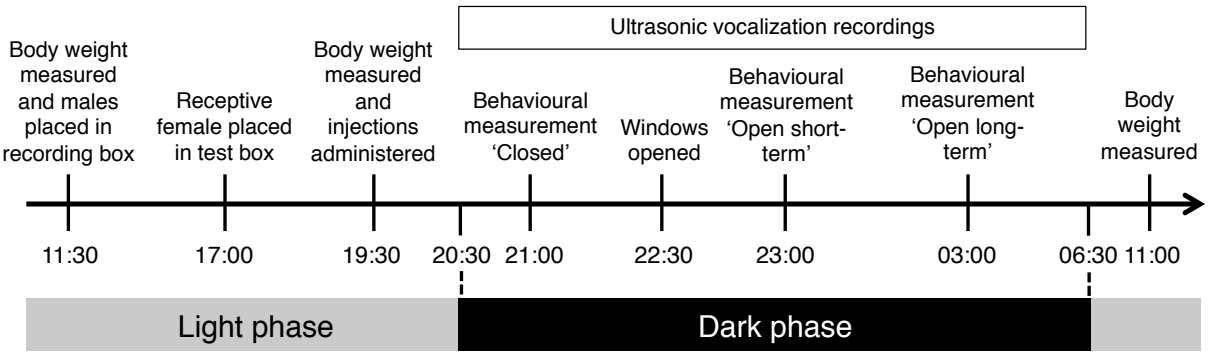
730

731 Figure A1



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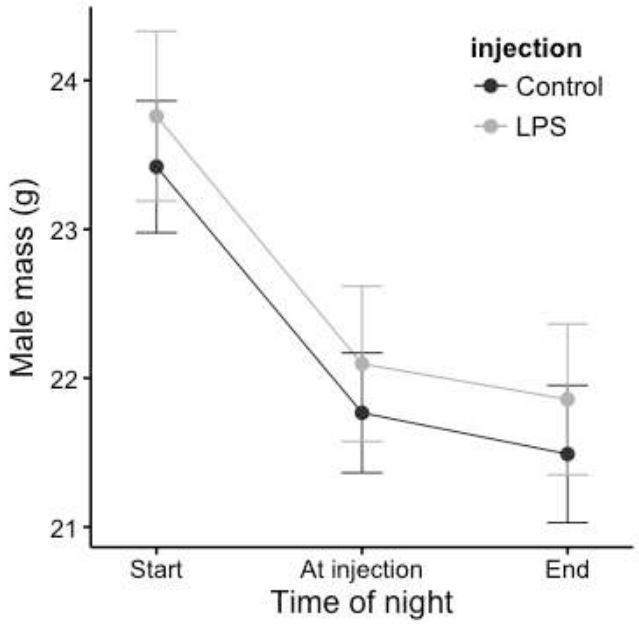
734 Figure A2



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